

A NON-DENATURING PROCESS TO PURIFY RECOMBINANT PROTEINS FROM PLANTS

FIELD OF THE INVENTION

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The present invention is in the area of biochemistry and protein technology and relates to improved methods of isolation and purification of heterologous proteins from transgenic plant material.

10 BACKGROUND

Protein based biopharmaceuticals show great promise in providing more specific and tissue specific, or cell specific drug treatments against serious diseases (for overview see "Recombinant Protein Drugs" Ed. P. Buckel 2001).

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Numerous examples in the prior art and applications have demonstrated the use of microorganisms such as bacteria, and animal cells for the production of such biopharmaceuticals, of which insulin is a notable example.

20 Many examples in the literature have demonstrated the utilization of transgenic plants or plant cell cultures for expression and manufacturing of high-value heterologous polypeptides or biopharmaceuticals. Such plant-based manufacturing process may be called molecular farming.

25 Production of valuable proteins can be made more economical by the use of plants as production organisms. The cultivation cost for plants used as host organisms for protein manufacturing can be considerably lower compared to most production systems based on bioreactors, such as prokaryotic production systems, animal cell cultivation and so forth. However, for all of the above
30 production systems, purification of heterologous proteins remains a demanding and costly task. Thus, for plant-based production systems, downstream processing generates most of the production costs in the manufacturing of high-value heterologous proteins.

35 Protein purification and isolation is a key process in downstream processing of proteins accumulated and produced in a variety of host organisms by the use of gene technology. The purification of proteins from the host organisms can be

quite laborious, complex and expensive. A variety of chromatographic strategies are used commercially for separation and purification of proteins of interest from production host organisms. The chromatographic strategies may rely on physicochemical differences between contaminating or endogenous proteins and the heterologous protein of interest, such as in size, solubility, charge, hydrophobicity, and affinity.

Combinations of chromatographic strategies consisting of multiple steps, require several expensive chromatography matrices and the necessary hardware consisting of columns, control units and so forth, and are accompanied by product yield-losses at each step, and consequently, economical losses. In addition to the chromatographic steps involved, downstream processing typically involves multiple filtering and centrifugation steps. As a result, the cost of purification and downstream processing may become prohibitive for the purification of a protein based biotechnological product. As for a number of protein-based products of lesser value, such as industrial proteins, the cost of downstream processing can be inhibitory for their use and marketing, resulting in crude and poorly defined products. For most biotechnology products purification costs are certainly a major proportion of the manufacturing costs.

The cost of specialized chromatography matrices effecting the separation of a protein of interest from contaminants is high as a result of complex coupling chemistry involved in their production. Some common chromatography methods are ion exchange chromatography, hydrophobic interaction chromatography and reversed phase chromatography. Ligands coupled to a matrix may include a variety of diethylaminoethyl- or quaternary aminoethyl groups as anion exchangers and carboxymethyl substituents and sulfonate groups and phosphates as cation exchangers of varying strength (Scopes, 1993), hydrophobic alkyl, phenyl or butyl groups coupled to crosslinked agarose matrices for hydrophobic interaction chromatography (Hydrophobic Interaction Chromatography (HIC) nr. 18-1020-90, Amersham Pharmacia Biotech), or hydrophobic n-alkyl groups as ligands grafted to a porous, insoluble beaded matrices composed of silica or synthetic organic polymers (Reversed phase Chromatography (RPC), Code nr. 18-11134-16, Amersham Pharmacia Biotech) The chemical complexity of these matrices may cause unwanted leaching of ligands and other substances from the matrix during a purification procedure, where necessary preventive measures, monitoring, or the removal of leachates from the protein of interest add to the already high cost of downstream processing.

Affinity chromatography is among the most powerful purification principles as it is based on specific affinity between an agent and a specific ligand, often mimicking a natural protein-ligand interaction. Several different kinds of affinity adsorbents

5 are available, some highly specific for a particular protein, others binding to classes of proteins rather than particular proteins. In many cases affinity chromatography implies the use of an immobilized ligand to an adsorbent that specifically selects out proteins binding to that ligand. The coupling of ligands to affinity adsorbents involves the use of coupling chemistry such as cyanogen
10 bromide-, tosyl-, or vinylsulfone-activation of adsorbents. The ligand coupled to the column matrix may or may not be of proteinaceous origin. Examples of the former are, but not limited to, immobilized protein A or protein G having affinity for γ -globulin, therefore being useful in the purification of antibodies, and lectins with affinity for glycoproteins. As an example of the latter, immobilized
15 glutathione coupled to matrix binds fusion proteins containing a glutathione S-transferase domain. Immobilized metal affinity chromatography (IMAC) is based on immobilization of metal-chelating ligands to a matrix and relies on the formation of weak coordinate bonds between metal ions immobilized on a column and basic groups on proteins, mainly histidine residues. Commercial cloning
20 vectors provide for cloning of a cDNA in frame with a string of histidine residues – a His-tag, that enables the purification of the resultant fusion protein with IMAC. Although widely used for small-scale purification of proteins, IMAC is a non-specific but selective method, as native histidine residues in contaminating
25 tags or binding domains are available in commercial expression vectors resulting in fusion proteins where the tag/binding domain binds the fusion protein to a ligand coupled onto a column matrix.

High specificity of protein binding can be achieved with these matrix-ligand
30 systems. In the cases mentioned above, complex coupling chemistry is involved to immobilize a ligand onto an inert matrix. Consequently, the cost of an affinity matrix can often become inhibitory to industrial scale applications of this powerful technique. Furthermore, as with most other types of chromatography methods, the stability of the coupling of the ligand to the matrix becomes an issue and
35 leaching is of great concern. Heavy metal leaching in IMAC can cause unacceptable and serious contamination in many sensitive purification processes for bioactive proteins, and may inactivate proteins being purified (Scopes 1993).

It is of particular relevance for the present invention that it is not uncommon that the binding affinity of a protein to its ligand is so strong that conditions for elution, to disrupt the ligand-protein binding, require drastic conditions that partly denature the valuable protein being purified. A non-limiting example of this is the elution of antibodies from Protein A-affinity matrix, requiring denaturing at low pH to release the antibody from the column. Including a denaturing step in a protein purification process is undesirable due to the risk of loss of activity of the purified protein, the addition of an extra step for refolding the protein and subsequent activity analysis requirements for the refolded protein product, and the added cost involved.

To enable the use of affinity-based chromatography for large scale purification from plants, it is highly desirable to develop a non-denaturing protein purification process that is simpler and more economic than the current measures commercially available, with less coupling chemistry involved and compatible with the quality requirements of the pharmaceutical industry standards.

Plant-based production of proteins shows great promise for large scale manufacturing of proteins in an economic manner, as has been shown by examples in literature (for overview see Hammond 1999). The cultivation costs involved in molecular farming with plants are considerably lower than with traditional bioreactor-based methods. Whereas upstream events in plant-based production look particularly promising, the downstream processing is facing the same challenges as the rest of the protein production industry.

Polysaccharides and polysaccharide binding proteins may be used in conjunction for the design of an affinity chromatography step (see, e.g., Boraston et al., 2001).

In an example of prior art (U.S. Patent No. 6,331,416) Shani et al. describe a method of expressing a recombinant protein with a polysaccharide binding domain that binds to the poorly defined cellulose in the host plant cell walls, and a protein purification process utilizing the affinity of this protein to host plant cellulose, resulting in a cell wall-protein complex that can be separated from soluble contaminating proteins. The strength of binding can be such that releasing the protein from the cellulosic host plant matter may require drastic conditions that denature the protein, having negative effects on the activity of the recombinant proteins being purified. Thus, although presenting a way to purify

proteins from plant material the harsh conditions required for disrupting the ligand/cell-wall binding involve complications and concerns comparable to those mentioned above for antibody-Protein A elution.

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The carbohydrate binding domain CBM9-2 is from the *Thermotoga maritima* Xylanase 10A (Winterhalter et al 1995: Mol. Microbiol. 15 (3), 431-444). The CBM9-2 genomic DNA sequence is available as GenBank Accession No. Z46264 and it belongs to the Family IX of CBM-s and has number of attractive properties
15 for high-resolution affinity purification, including non-denaturing eluting conditions using 1M glucose as a eluent, and high specific affinity for amorphous as well as crystalline celluloses (Boraston et al. 2001: Biochemistry 40, 6240-6247).

- 20 Recovery and purification of expressed recombinant proteins from transgenic plants is probably the most critical factor in establishing plants as a practical alternative system for protein production. It is essential that the number of processing steps be minimized and that each step be carried out at much higher efficiencies. (Moloney 2000)

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This emphasizes a recognized need for a downstream process for purification of heterologous proteins from transgenic plant material that is efficient, simple, and economical. Furthermore, there is a need for such a downstream process consisting of gentle, non-denaturing conditions for the protein of interest, (In
30 particular, specific affinity purification methods with gentle elution conditions) in order to secure bioactivity of the protein of interest, and improve yields.

- A non-denaturing protein purification process free from the limitations detailed above could significantly lower the production cost involved in the production of
35 biopharmaceuticals from plants, and would be enabling for the purification of heterologous proteins of value for which downstream processing has been prohibitively complex and costly.

SUMMARY AND OBJECTS OF THE INVENTION

The primary objective of present invention is to provide an improved, non-denaturing method for protein purification of high-value heterologous proteins produced in plants, plant derived tissue or plant cells.

The invention aims to reduce the cost and improve the quality of downstream processing of heterologous proteins produced in plants.

An important step in the purification process is the separation of the protein of interest as a CBM-fusion protein from cell-wall fragments and other poorly defined plant-derived solids as the CBM-fusion protein of the invention does not bind to these components. This can be done separately prior to an affinity chromatography step or simultaneously with an affinity chromatography step.

In a first aspect, the invention provides a non-denaturing method for production and purification of a soluble heterologous fusion protein comprising a cellulose binding module (CBM), from transgenic plants or transgenic plant cells expressing said fusion protein, the method comprising:

disrupting the transgenic plant material;

adding an extraction liquid to the plant material, thereby creating a mixture of soluble and insoluble plant material, so as to extract the soluble fusion protein from said disrupted plant material to the liquid phase to obtain a protein extract;

separating the insoluble plant material, comprising cell-wall material and solids, from said protein extract comprising said fusion protein of interest;

contacting said protein extract to a polysaccharide matrix which binds to said fusion protein;

washing the matrix with the bound fusion protein with one or more suitable aqueous solutions such as one or more buffer solutions, i.e., the washing may be preformed in one step, with a gradient or as a sequence of different washing solutions; and

eluting the fusion protein from said polysaccharide matrix by adjusting conditions effecting the release of said fusion protein from the matrix under characteristically mild conditions.

Typically, the transgenic plant or plant cell is selected from the group of dicotyledonous plants and monocotyledonous plants, and in preferred embodiments said plant cell or transgenic plant is from the group of tobacco, rape seed, soy bean, lettuce, alfalfa, barley, maize, wheat, oat and rice.

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The separation step comprises in some embodiments a method selected from expanded bed adsorption (EBA), packed mode chromatography, precipitation, filtration, centrifugation, or any combination thereof.

- 10 The affinity binding step binding the fusion protein to a polysaccharide matrix preferably comprises a chromatography step.

However, in certain useful embodiments, said separation of cell-wall fragments and other poorly defined plant-derived solids from the protein of interest – the
15 CBM-fusion protein, and the affinity binding of the CBM fusion protein to a polysaccharide matrix can be done in a single powerful purification step using Expanded Bed Adsorption chromatography (EBA) with a suitable, inexpensive polysaccharide matrix. This feature streamlines and improves the economy of the downstream processing.

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In advantageous embodiments of the present invention, the polysaccharide matrix comprises cellulose, and preferably pharmaceutically compatible cellulose. Such a well-defined pharmaceutical grade cellulosic matrix to which the CBM-fusion protein binds allows various high-end uses of the purified heterologous
25 protein. A useful pharmaceutically compatible cellulose material for use as the polysaccharide matrix comprises Avicel™ (FMC Corporation, PA, USA).

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The polysaccharide matrix used for affinity chromatography according to the invention requires no complex coupling chemistry or immobilization of potentially leaching ligands. The polysaccharide matrix provides both structural support and rigidity while constituting the affinity adsorbent itself. Thereby, more economical and safer protein purification is enabled for plant derived heterologous proteins.

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It is still an advantage of this invention that the process described is amenable for different polysaccharide matrices of differing qualities all according to the different end-use of the purified heterologous proteins e.g. in for example agriculture, chemical industry or pharmaceutical industry. An affinity adsorbent made out of polysaccharide of pharmaceutical grade is a bulk material within the

pharmaceutical industry and is considerably less expensive than any commercially available affinity chromatography media. Thus, an affinity matrix of very high quality can be made economical using the process described by this invention, enabling more economical downstream processing of high-value proteins from plant derived material.

It is a further advantage of the present invention that once the plant derived CBM-fusion protein is bound to the polysaccharide chromatography matrix, and after washing the matrix to remove any contaminating endogenous plant proteins, the fusion protein can be eluted from the column using non-denaturing, mild conditions typically under neutral or acidic conditions and preferably with the addition of soluble carbohydrates (sugars), that preserve the activity and structure of any fusion partner protein attached to CBM. The sugars compete with cellulose for the binding site of the CBM and a suitable concentration will release substantially all of the bound CBM fusion protein.

It follows that the preferred CBM-s that are used in the methods of the invention and fused to the heterologous protein of interest are such CBM-s that have desired binding characteristics to allow sufficiently strong binding to a suitable polysaccharide matrix to obtain a high yield of bound CBM fusion protein, and releasing by such mild conditions as described above. CBM9-2 has been found to have these desired characteristics. The use of other CBM-s with such characteristics is also within the scope of the invention. Such CBM-s may be found e.g. by searching available gene databases for sequences encoding CBM having desired characteristics, e.g. sequence motifs found to be similar to motifs in CBM9-2 that are important for the binding characteristics. Also, existing CBM-s may be modified with point mutation techniques well known in the art to modify their binding characteristics in order to obtain suitable CBM-s according to the invention.

After the fusion protein has been eluted from the polysaccharide affinity matrix it may optionally be subjected to one or more further purification or isolation steps, depending on the desired form and use of the protein.

In useful embodiments, the transgenic plant or plant cell comprises a nucleic acid sequence encoding for a CBM, preferably the CBM is heat-stable and remains soluble at elevated temperatures. The term heat-stable in this context indicates that the protein remains soluble, correctly folded and active at elevated

temperatures, i.e. temperatures above about 25°C, and typically above about 37°C, including the range of 40°C -100°C.

Genes encoding such preferred CBM may be obtained from thermophilic organisms, including thermophilic bacteria, algae and fungus and introduced into the host plant or plant cell in such a way as to express a fusion protein comprising said CBM. The term thermophilic refers herein to organisms with optimal growth temperature over 40°C. A preferred CBM is coded for by the xylanase10A gene from *Thermotoga maritima*, preferably the region within the host plant or plant cell that codes for a CBM is a region of said gene. Said region coding for a CBM may in certain embodiments comprise a sequence depicted as SEQ ID NO: 1, or a nucleic acid sequence encoding the same amino acid sequence, or a sequence encoding an amino acid sequence with substantial sequence identity to said amino acid sequence.

It may be useful in some embodiments of the invention to heat the protein extract comprising the soluble fusion protein, such as to a temperature in the range of 37°C and 100°C, e.g. a temperature in the range of 50-80°C, for a period of time such as in the range of from 1 min to 120 minutes during the process. For this purpose, heat-stable CBM such as from thermophilic sources is particularly useful. In some useful embodiments, the heat-stable CBM may promote solubility of attached heterologous protein at elevated temperatures, a feature that may be used for CBM-fusion protein enrichment of an extract containing other heat labile plant proteins. During such heating, a part of the endogenous plant proteins may become inactivated and/or denatured and may thus readily be separated from the protein extract. Said heated extract may preferably be subjected to the process step comprising expanded bed adsorption with a polysaccharide matrix for the simultaneous separation of solids and affinity binding of said CBM fusion protein from the heated extract.

In a highly useful embodiment of the invention, said fusion protein comprises a CBM and a heterologous polypeptide of interest intercepted by a stretch of amino acids comprising a proteolytic cleavage site, preferably a proteolytic cleavage site recognized and cleaved by a specific protease. By having such a site, the CBM can readily be cleaved off the fusion partner in the fusion protein to obtain the desired purified heterologous protein without the accompanying CBM. Detailed description for such successful purification involving a specific protease is disclosed in applicant's co-pending application "A process for proteolytic cleavage and

purification of recombinant proteins" which application is filed simultaneously with this application and is incorporated herein in full by reference.

5 The present invention successfully addresses the short-comings of downstream processing involved in of heterologous protein production at large scale, for purposes such as, but not limited to, agriculture, chemical industry and the production of protein-based pharmaceuticals. In particular, it provides a novel process of separating CBM-fusion proteins from biomass such as plant-derived cellulosic material, with fewer processing steps involved, taking advantage of a
10 a safer and more economical affinity chromatography principle amenable for use within the pharmaceutical industry, gentle elution conditions maintaining the activity of high-value heterologous proteins, and in applicant's co-pending application " A process for proteolytic cleavage and purification of recombinant proteins" comprising a process step enabling the recycling of a specific high-value
15 protease in the process.

BRIEF DESCRIPTION OF DRAWINGS

20 Figure 1 is a schematic diagram of a preferred embodiment of the purification process.

Figure 2 demonstrates the results obtained from Example 1 by SDS-PAGE analysis. Lane 1: molecular weight size markers, lane 2: purified CBM9-2, lane 3:
25 washed solids from milled barley seed, lane 4: supernatant after biomass interaction, lane 5: first wash with low salt buffer, lane 6: fifth wash with high salt buffer.

Figure 3 demonstrates the successful purification of CBM9-2 from milled barley seed extract according to the method of this invention, as described in detail in
30 Example 2. The figure shows the chromatographic profile of an expanded bed absorption (EBA) column with 200 mL cellulose.

Figure 4 demonstrates the results obtained from Example 3, Heat stability and
35 enrichment of CBM9-2 in extracts. The figure shows a SDS-PAGE analysis of heat stability of CBM9-2 in barley extracts subjected to different temperatures. Lane 1: molecular weight size markers 10-200 kDa, lane 2: barley seed extract at room temperature (RT), lane 3: purified CBM9-2 (RT), lane 4: CBM9-2 + barley seed

extract (50°C), lane 5: CBM9-2 + barley seed extract (60°C), lane 6: CBM9-2 + barley seed extract (70°C), lane 7: CBM9-2 + barley seed extract (90°C).

Figure 5 demonstrates results obtained from Example 4. The graph shows ELISA readings of a control sample and a minimal reading of sample containing a heterologous protein of interest (HoxB4) fused to CBM9-2 and purified according to the down-scaled version of the purification process described herein. The columns show the measured ELISA values for (a) elution buffer, (b) fusion protein extracted from transgenic seeds, and (c) protein extracted from non-transgenic seeds.

DETAILED DESCRIPTION OF PRESENT INVENTION

Herein below, the present invention will be described in more detail.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood and used by one of skill in the art to which this invention belongs.

The term "polypeptide" used herein refers to any polymer of amino acids, being monomeric or multimeric, and does not refer to a specific length of a polymer of amino acids. Thus, for example, the terms peptide, oligopeptide, protein, and enzyme are included within the definition of polypeptide. This term also includes polypeptides with post-expression modifications such as for example, glycosylations, acetylations, phosphorylations and the like.

The term "heterologous polypeptide of interest" or "polypeptide of interest" used herein refers to any polypeptide intended for expression in plant-cells or plant tissue using the methods or compositions of the present invention. As non-limiting examples, pharmacological polypeptides (e.g., for medical uses) or industrial polypeptides (e.g. enzymes) can be produced according to the present invention.

The term "downstream processing" refers to the isolation and purification of a biotechnological product to a form suitable for its intended use.

The term "fusion partner" refers herein to a heterologous protein linked to CBM.

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The term "CBM fusion protein" refers to a molecule consisting of a CBM linked to a heterologous protein, and in the context it is put forward in this invention, a molecule without a proteolytic cleavage site, unless described otherwise.

- 5 The term "operably linked" refers to a functional linkage between a promoter (nucleic acid expression control sequence, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the promoter directs transcription of the nucleic acid corresponding to the second sequence.
- 10 The term "denatured" refers to a condition of a protein where the native structure, and consequently the activity of the protein is disrupted, and the protein is unfolded or incorrectly folded changing its native three-dimensional structure.
- 15 The term "expression" and "production" refer to the biosynthesis of a gene product, including the transcription and translation of said gene product.

"Molecular farming" refers to the operation of using plants of any kind in open fields or in closed facility to express and produce heterologous proteins in their
20 tissue

The term "transgenic" refers to a any cell, cell line, tissue plant part, organ or organism into which a non-native nucleic acid sequence has been introduced, and therefore altering its genotype, as is progeny thereof in which the non-native
25 nucleic acid is present. Typically, the non-native nucleic acid sequence was introduced into the genotype by a process of genetic engineering, or was introduced into the genotype of a parent cell or plant by such a process and is subsequently transferred to later generations by sexual crosses or asexual propagation.

- 30 "Substantial sequence identity" indicates in the context herein at least 50% sequence identity and more preferably at least 60% such at least 70 sequence identity, such as at least 80% and preferably at least 90% sequence identity, such as at least 95% or 99% sequence identity. Algorithms for sequence analysis
35 are known in the art, such as BLAST, described in Altschul et al., *J. Mol. Biol.* (1990) 215:403-10. Generally, the default settings with respect to, e.g. "scoring matrix" and "gap penalty" will be used for alignment.

The term "transformation" or "transformed" refers to the introduction of a nucleic acid sequence into the DNA genome of a host organism, irrespective of the techniques used for the introduction of the nucleic acid fragment into the host cell.

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"Thermophilic" refers to an organism with optimal growth temperature over 45°C.

The term "GMP" (good manufacturing practice) dictates the manner in which biopharmaceuticals and other drugs and medical devices are produced. GMP requirements include standard operating procedures, sterile conditions, validation of materials and equipment and trained personnel.

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Monocotyledonous and dicotyledonous plants that can be genetically manipulated can be used in the present invention. Preferably the plant is a monocotyledonous, more preferably barley, and most preferably the barley *Hordeum vulgare*. A plant that can be genetically transformed is a plant into which non-native DNA sequence, including DNA sequence for a coding region, can be introduced, expressed, stably maintained, and transmitted to subsequent generations of progeny. Genetic manipulation and transformation methods have been used to produce barley plants that are using herbicides including, for instance, bialaphos or basta, or antibiotic, such as hygromycin, as selectable markers.

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METHODS

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Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

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Although only preferred embodiments of the invention are specifically illustrated, numerous modifications and variations in the invention as described in the above examples are expected to occur to those skilled in the art, without departing from the spirit and intended scope of the invention.

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Referring to Fig. 1 illustrating a schematic diagram of the purification process the process can be described as follows:

(1) The starting material for the process is a transgenic plant, material
5 derived from transgenic plants or transgenic plant cells, including, but
not limited to, suspension cultures of plant cells as well as
undifferentiated cells of calluses. The material is transgenic in a way that
it expresses in a controlled manner a heterologous gene(s) operably
10 linked to a CBM open reading frame, that have been introduced to the
plant cells through processes known to a person skilled in the art, such
as, but not limited to, Agrobacterium-mediated transformation or particle
bombardment-mediated transformation or plant viral vector-mediated
transformation. The starting material is preferably selected on the basis
15 of satisfactory expression levels of the fusion protein, as deemed by an
analysis of RNA or protein levels by a person skilled in the art, prior to
the initiation of the process described by the invention.

(2) Disruption of the transgenic material is accomplished by any method
20 known to a person skilled in the art, that results in homogenization of
the plant tissue and plant cells, in a dry or wetted state. A variety of
methods can be chosen from, that suit the source of the plant material.
Thus, for seeds, milling is a good way of disrupting the transgenic plant
tissue, while for leaves and softer green tissue homogenizing can be
25 accomplished with equipment such as, but not limited to, Waring
blender, Sorvall Omnimixer or Polytron homogenizers. The equipment for
disruption of plant tissue and plant cells is commercially available and
easy to scale up as required. General methods of extraction of proteins
from plant sources are described by G. Paul Powell in a publication
30 edited by S. Roe, Protein purification applications, 2nd edition (2001). A
simple and successful method of extraction of soluble proteins from plant
sources is the addition of simple buffers like Low salt buffer to the
disrupted, homogenized plant tissue, with thorough mixing. Precautions
against oxidative tanning, such as addition of polyvinyl pyrrolidone (1%
35 w/v) are usually sufficient to optimize purification from most plant
tissues in order to sequester phenolics from the plant tissue that
otherwise could have negative effects on the heterologous protein to be
purified. Proteolysis does not always cause problems with plant sources.
If, however, proteolysis is a concern, protease inhibitors, such as, e.g.,

serine-, cysteine- and metalloprotease inhibitors can be added to the extraction buffer. The disruption of the plant material can be done in the presence or absence of a buffered solution. The extraction solution may or may not contain reducing agents such as, but not limited to 2-mercaptoethanol or dithiothreitol (DTT). Soluble plant proteins will be present in the liquid phase together with the CBM-fusion proteins

(3) Mixing of liquid with plant material is essential to extract the water-soluble fusion protein to the liquid phase. The liquid added may or may not contain buffering agents to control pH, preferably within the range of about 5.2 to 8.3, it may or may not contain any reducing agents or sequestering agents as described in (2) accordingly to the protein of interest. In its simplest form, the liquid can be water. After thorough mixing of liquid with the disrupted and homogenized plant material, the liquid phase now contains the CBM-fusion protein.

(4) (A) Depending to some degree on the level of homogenization the mixture of disrupted plant material and extraction liquid can be applied directly to an Expanded Bed Adsorption (EBA) column. In this approach the mixture is applied to the column as a stream of fluid through an expanded bed of affinity adsorption matrix of polysaccharide nature. During the streaming through the column the fusion protein in the liquid phase is exposed to the polysaccharide matrix and is selectively adsorbed through the selective affinity of the CBM to the polysaccharide adsorbent media. Particles such as, but not limited to, cell wall fragments and other solids, together with any soluble plant proteins are flushed through the EBA column in the flow-through liquid.

4(B) Alternatively, a majority of the solids in the mixture can be separated from the liquid prior to the affinity binding step through a variety of methods known to a person skilled in the art, these include, but are not limited to, precipitation, filtration, centrifugation, and sedimentation. As described hereinabove, the solids are discarded and the liquid containing the CBM-fusion protein is subjected to an optional heating step 4(C) or applied directly to the affinity binding step (5).

4(C) Heating of the mixture or liquid prior to affinity step (5) is optional, but may act as an additional purifying step in cases where the CBM-fusion protein as a whole remains soluble at elevated temperatures, while soluble plant proteins may denature and precipitate and endogenous plant proteases may be inactivated through heat. For this purpose, the heating procedure, taking into account the nature of the

CBM-fusion protein, may involve heating in the range of 50°C to 100°C, for a period of time in the range of 2 min to 60 minutes during the process. In these embodiments CBM of thermophilic origin are particularly beneficial.

5 (5) Affinity binding to matrix. The liquid protein extract containing the plant derived CBM-fusion protein is brought into contact with the polysaccharide matrix towards which the CBM has affinity for. The contact can be effected in various ways, such as, but not limited to, chromatography columns packed with the polysaccharide matrix where
10 the liquid is run through the column in either packed or expanded mode, referring to the density of the polysaccharide matrix, or it can be effected in batch mode where the polysaccharide matrix is mixed together with the liquid in a suitable container, with the subsequent recovery of the matrix and the adsorbed CBM-fusion protein. The
15 polysaccharide matrix can be of cellulosic origin such as, but not limited to, Avicel, or it can be of xylanoic origin, such as insoluble xylan. The binding specificity and thermodynamics of CBM9-2 have been studied in detail in a recent publication by Boraston et. al. (2001). It has however surprisingly been found by the present inventors, in contrast to what is
20 indicated in the prior art, that following the methods of the present invention as described herein, CBM9-2 does not bind to plant cell wall components but becomes readily soluble, while retaining good specific binding to a polysaccharide matrix such as used in the affinity adsorption step herein (5). As described herein above this surprising quality
25 introduces several advantages to downstream processing of plant derived CBM-fusion proteins to the extent that a greatly improved downstream processing method is provided.

(6) Washing of the matrix. The polysaccharide affinity adsorbent with CBM-fusion protein bound to it can be washed with several column
30 volumes (relevant quantitative term if the affinity matrix is placed in a chromatography column) of an aqueous solution (e.g. water) or buffer, such as, but not limited to phosphate buffered saline or Tris-based buffers. To improve the efficiency of the washing step, the composition of the washing buffer may be adjusted by means such as, but not limited
35 to, several column volumes of stepwise changes or a gradient of salt concentration or detergent, used to release weakly but nonspecifically bound contaminating proteins from the matrix.

(7) Release of fusion protein from matrix. By using CBM-s with the desired binding characteristics as described herein, elution of the CBM-fusion protein from the affinity matrix can be effected with the exposure of the CBM-fusion protein to competing saccharides such as, but not limited to glucose, galactose, lactose, maltose, and cellobiose. Any of these or other similar saccharides, or a combination thereof, can be added in a suitable amount, such as in the range of 1mM to 1 M concentration to an elution buffer such as, e.g., phosphate buffered saline or Tris-based buffers, for the elution step. The saccharide concentration can be e.g. in the range 25 mM to 1 M, such as in the range 50 – 500 mM. These saccharides are commercially available as low-cost bulk chemicals, further improving the overall economy of the downstream processing according to the invention.

(8) Isolation/purification of fusion protein. Further purification/isolation of the CBM-fusion protein may be advantageous or required be in some instances. Such further isolation can be accomplished with any of the commonly available chromatographic procedures known to a person skilled in the art, such as, but not limited to, ion exchange chromatography or size exclusion chromatography.

(9) Final product. The final product is in this case a CBM-fusion protein in a highly purified form, ready for optional proteolytic cleavage of the CBM-fusion protein or further purification/isolation that may be required or be advantageous in some instances. This further isolation can be a chromatography step, such as, e.g., ion exchange chromatography or size exclusion chromatography.

The highly purified form is ready for further formulation and packaging in its final form, if necessary.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which is not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

EXAMPLE 1

Biomass interaction study: CBM9-2 and milled barley seed

Dried barley seed was finely ground in a Retsch mill to a fine flour. 1 g of milled seed was used for the extraction of water-soluble components from the seed with 5 ml of low salt buffer (50 mM potassium phosphate buffer pH 7.02), as a means to rid the sample of all water-soluble components that might interfere with the biomass interaction study. The mixture was vortexed and tumbled for 5 minutes to ensure thorough mixing of the liquid and the milled barley seed material.

Following this mixing, the samples were centrifuged at 5000 x g for 4 minutes to pellet the solids. After centrifugation, the supernatant was discarded. This procedure was repeated 3 times with low salt buffer, with the supernatant discarded after each centrifugation. Then the washing procedure was repeated 3 times with high salt buffer (50 mM potassium phosphate buffer pH 7.02, 1 M NaCl), the supernatant discarded as before. The resulting washed solids representing for the most part plant cell-wall fragments and insoluble starch.

The washed solids were equilibrated with 3 times washing with low salt buffer as described above, to obtain same conditions that favor affinity binding of CBM9-2 to cellulosic matrix in affinity chromatography. A representative sample of the solids before biomass interaction study was taken for SDS-PAGE analysis (lane 3). 10 µl of bacterially produced CBM9-2 purified on cellulose (Avicel™)-affinity column (O.D. @280 nm 0,394), were taken for later SDS-PAGE (lane 2). The purified CBM9-2 had previously been subjected to repeated (4x) dilution and concentration in a ultrafiltration module as a proven method for desorbing any bound glucose from the CBM9-2, so as to regain the cellulose binding affinity characteristic of the protein.

2 ml of the purified CBM9-2 were added to the washed, equilibrated solids derived from milled barley seed and the mixture incubated while shaking for 60 minutes at room temperature. After incubation, the mixture was spun down at 5000 x g for 10 minutes, and the supernatant subsequently clarified with centrifugation at 13.000 x g for 5 minutes. 10 µl of the clarified supernatant from the biomass interaction was taken for SDS-PAGE analysis (lane 4). Subsequently the pellet consisting of the milled barley seed solids, was washed 5 times with low salt buffer and subsequently 5 times with high salt buffer as described above. 10 µl of the first low salt buffer wash (lane 5) and the fifth low salt buffer wash (lane 6), were prepared for subsequent SDS-PAGE analysis. To elute any bound CBM9-2 from the milled barley seed solids, 1 ml of elution buffer (1 M glucose in 50 mM KPO₄, pH 7.02) was added to the solids and incubated during mixing for 15 minutes, before centrifugation at 5000 x g for 5 minutes and removal of the

supernatant (eluate). A 10 µl sample of the eluate was prepared for SDS-PAGE analysis (lane 7). A representative sample from the solids after the biomass interaction study and elution was taken for SDS-PAGE analysis (lane 8). The samples from the biomass interaction assay prepared for SDS-PAGE were run on 12.5 % SDS-PAGE gels (PhastGels homogenous 12,5) using PhastSystem (Amersham Pharmacia Biotech). After completion of the run the gel was stained with Coomassie Blue R-250, and destained. The results are illustrated in FIG. 2.

These results demonstrate that CBM9-2 does not bind significantly to plant derived cell-wall fragments or other insoluble solids from milled barley seed.

EXAMPLE 2

Purification of CBM9-2 from milled barley seed extract

Barley seeds were milled to finely ground flour using commercially available mill (Aarslev Maskinfabrik, Erhvervsvangen 11, 5792 Aarslev, DK). The resulting barley flour was wetted in Low salt buffer (50 mM potassium phosphate buffer pH 7.02) in volume-ratios 2:3, barley flour:buffer, respectively. The liquid was mixed thoroughly with the flour in a vessel and allowed to sediment overnight at 4°C. CBM9-2 purified from bacteria was added to the barley seed-supernatant. The next day the spiked supernatant (100 ml) containing CBM9-2 was fed to a Streamline 25 (Amersham Biotech) chromatography column containing cellulose (Avicel™). The feed application was done at flowrate 184 cm/h, in expanded bed mode, followed by a washing step with 5 column volumes high salt buffer (1 M NaCl in 50 mM KPO₄, pH 7.02), followed by 5 column volumes of low salt buffer (50 mM KPO₄, pH 7.02). The expanded column bed was allowed to sediment (sedimented bed height = 20 cm) and elution was performed at 92 cm/h with 300 ml of elution buffer: 1 M glucose in 50 mM KPO₄, pH 7.02). The elution conditions resulted in a small peak containing the CBM9-2 protein (see Fig. 3). This showed that using the procedure described hereinabove that firstly; CBM9-2 remains in solution unattached to cell-wall fragments and other poorly defined solids from milled barley seed, secondly; it is possible to use polysaccharidic affinity chromatography as described by this invention to capture CBM 9-2 from milled barley-seed extract, thirdly; this can be done by using well defined pharmaceutical grade cellulose (Avicel) as a matrix, and fourthly; the affinity chromatography step can be done in expanded bed mode as described by the invention, fifthly; the CBM9-2 purified from barley seed-extract can be eluted of

the matrix under gentle conditions avoiding any denaturing steps, as described by this invention. The very same conditions and procedure as described hereinabove, can be applied to purify CBM9-2-fusion proteins from transgenic milled seed.

The polysaccharidic affinity chromatography described is also valid for the

- 5 separation of protease-CBM and excised CBM from the protein of interest after a proteolytic cleavage reaction, as described by this invention.

EXAMPLE 3

Heat stability and enrichment of CBM9-2 in extracts.

10

1.5 grams of milled barley were dissolved in 7.5 ml of Low salt buffer (50 mM KPO₄, pH 7.02). The solution was continuously mixed in a tumbler for 1 hr. And spun down at 6000 rpm for 10 minutes. The supernatant (extract) was measured for protein content with Bradford assay and found to contain 1,93 mg/ml of
15 soluble seed proteins. 350 µl of extract were mixed with 350 µl of purified CBM9-2 protein (0.238 mg/ml) and aliquoted to Eppendorf tubes that were subsequently exposed to different temperatures (room temperature, 50°C, 60°C, 70°C and 90°C) in a water bath for 10 minutes. After heat treatment the samples were spun down at 11.000 rpm for 10 minutes and samples from the different
20 heat treatments analysed by SDS-PAGE. The samples from the heat stability test were prepared for SDS-PAGE and run on 12.5 % SDS-PAGE gels (PhastGels homogenous 12,5) using PhastSystem (Amersham Pharmacia Biotech). After completion of the run the gel was stained with Coomassie Blue R-250, and destained. The results are illustrated in FIG. 4. Further tests on the CBM9-2
25 exposed to heat confirmed that its ability to bind to cellulose was not adversely affected in anyway by the exposure to heat.

The results show that while soluble barley seed proteins were gradually lost from solution CBM9-2 remained soluble during the entire temperature range. This shows heat may be advantageously applied for the purpose of enrichment of CBM
30 and CBM-fusion proteins during extraction stage of the purification process described by this present invention.

EXAMPLE 4

Purification of heterologous protein attached to CBM9-2 from milled, single barley
35 seed extract.

In a down-scaled version of the purification procedure described hereinabove, single seeds of transgenic barley plants expressing heterologous protein of

at room temperature for a period in the range of 30 sek to 5 min. When blue color had developed, 100 µl of 0.2 M Sulfuric acid was added. The color turned yellow, and the absorbance was measured in a microplate reader at 450 nm.

5 Results from an ELISA analysis of a single seed extraction from individual transgenic barley seed are shown in Fig. 5.

The ELISA analysis, previously shown to be specific for CBM9-2, shows firstly; that heterologous fusion protein accumulation in individual seeds can be achieved and verified; that the procedure described hereinabove works for the isolation of
10 a heterologous fusion protein with cleavage site, even at small scale, supporting the scaleability of the purification process; it shows that heterologous protein of interest attached to CBM9-2 is successfully maintained in solution during extraction and not lost due to binding to poorly defined plant cell wall cellulose; it shows that the heterologous fusion protein is not exposed to denaturation during
15 the chromatography process, as it is recognized after elution by the specific antibodies without any renaturation steps being involved, emphasizing the advantage of the present invention to several other harsher affinity chromatography procedures, as discussed hereinabove; that the purification procedure described hereinabove is applicable to any heterologous fusion protein
20 attached to CBM9-2.

EXAMPLE 5

Purification and activity measurements of CBM-protease

25 In order to produce a site-specific protease with a CBM9-2 tag attached to it the following procedure was followed:

Agrobacterium tumefaciens strain AGLO was constructed to contain a binary plasmid carrying an expression construct composed of a constitutive promoter in front of enterokinase cDNA to which was attached cDNA corresponding to CBM9-2, a signal sequence for targeting to Endoplasmic reticulum (ER) and a retention
30 signal to maintain the protein in the ER. This agrobacterium strain was grown in YEB media under selection conditions, first in 10 ml for 2 days at 28°C up to O.D. 600 of 0.8. The small culture is diluted 1:50 to 500 ml culture containing 20 µM acetosyringone for 2-3 days at 28°C and vigorous shaking up to O.D. 600 nm of 2.5. The bacteria was spun down at 6,000 rpm for 10 minutes and resuspended
35 in MS-solution (containing 55 g/l sucrose) to OD₆₀₀ 2.5. Acetosyringone was added (10 mM), for final concentration of 200 µM. Bacterial suspension was then

kept at room temperature for 1 hour and Tween-20 (10%) added, for final concentration of 0.005%.

For transient expression of CBM-protease in lettuce plants were submerged into a bowl containing the Agrobacterium bacterial suspension for 15 seconds.

- 5 Subsequently the plants were placed in a vacuum chamber and 0.4 bar pressure was applied for 20 minutes, after which air inlet was opened to equalize pressure rapidly. The excess bacteria on leaf surface was washed off with successive dipping into bowls of tap water. The lettuce plants were placed in a growth chamber with 16 hrs day/8 hrs night light period at 22°C, for 4 days.
- 10 The plants were harvested by excising the leafy tissue and subsequently frozen and kept at 86°C. The plants were homogenized using mortar and liquid nitrogen until a very fine powder was obtained. The powdered lettuce leaf material was extracted by the addition of 1.2:1 (vol:vol) low-salt extraction buffer and lettuce powder respectively, and proteins extracted for 30 minutes with occasional
- 15 mixing at room temperature.

The extract was subsequently centrifuged at 6000 rpm for 20 minutes to separate solid material and cell wall fragments from liquid phase. The supernatant was decanted and spun again as previously described. The clear supernatant was fed onto a packed bed column containing cellulosic matrix (Avicel™) as described

20 hereinabove. The CBM-protease attached specifically to the cellulosic matrix and after washing the column with 5 column volumes of high salt and low salt washing buffers, respectively, the CBM-protease was eluted off the column under mild, non-denaturing conditions, i.e. with 1 M glucose solution in a single peak.

- 25 The peak was subsequently concentrated using Millipore concentrators (Ultrafree-15 – Biomax-5).

- Enterokinase activity is assayed using specific synthetic substrate according to a standard approach (Grant & Hermon-Taylor, 1979): Synthetic substrate: Gly-Asp-Asp-Asp-Asp-Lys-β-naphthylamide (GD₄K-na); Assay conditions 37°C. Reaction volume is 1.5 ml. The reaction mixture consists of :25 μl 10 mM GD₄K-na (0.5
- 30 mM), 125 μl 100 mM Tris-HCl, pH 8.4 (25 mM), 50 μl 100% DMSO (10%), 50 μl 100 mM CaCl₂ (10 mM), (20-100) μl enterokinase, 250 μl distilled H₂O –(20-100) μl. The rate of β-naphthylamine formation was determined from the increment of fluorescence between λ_{ex}=337 nm and λ_{em}=420 nm. This was monitored continuously for 5 min.

The results from the activity measurements showed that CBM-enterokinase produced and purified as described, was active; Enterokinase activity was measured to be 442,7 cps/min/ μ g compared to blank 0,0001 cps/min/ μ g.

The example shows firstly; that a protease-CBM can be produced in plants, in this case transiently in lettuce, and that CBM-protease can be isolated and purified successfully using the purification procedure described hereinabove. It further shows that the CBM9-2 affinity tag of the fusion protein is fully functional; that the CBM-protease effectively to cellulosic matrix and It can be eluted off the matrix under the mild elution conditions described by the invention, and the eluted protease is shown to be fully active. The enzymatically active purified product provides in itself evidence for the non-denaturing properties of the purification process, as enzyme activity is particularly sensitive for partial or full denaturation, which easily results in loss of activity. This shows effectively that all components of the Invention are functional and that their behaviour and performance is such that they can easily be applied in the manner described by the invention hereinabove, resulting in a process constituting a major improvement in specificity, economy and efficiency of downstream processing of heterologous proteins of any source.

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